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(57) Abstract

This invention relates to a novel phytase produced from novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and more precisely, to a novel strain *Bacillus sp.* DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.

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DS11 (KCTC 0231BP), NOVEL *BACILLUS SP.* STRAIN AND NOVEL PHYTASE PRODUCED BY IT

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a novel phytase produced from novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and more precisely, to a novel strain *Bacillus sp.* DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.

Description of the Prior Art

Phytase is an enzyme to degrade phytic acid into phosphate and phosphate inositol. 50 to 70% of phosphate in grain used as livestock feeds exists in form of phytic acid but phytase is not present in monogastric animals such as hens and hogs, resulting in low phosphate availibility.

Therefore undigested phytic acid(phytictaine) released to a water source became one of the serious environment contamination sources causing to eutrophication in small lakes or tides. With above consideration, because monogastric animals can't utilize phytic acid in their intestine phytic acid chelates to water due to chelation with a trace amount of minerals, amino acids and vitamins which are very important to the metabolism of livestock. These formed water-insoluble, undigestable chelate complexes released to feces change the environmental ecosystem to induce a serious environmental pollution.

In view of these situations, the application of phytase into the livestock feeds will reduce the supply of inorganic phosphate due to increase of phosphate bioavailibility in livestock, leading to economic benefits, and improving the availibility of phosphate, and other bioactive substances, leading to reduction of the environmental contamination.

By these reasons, the utilization of phytase in livestock is very important. A law regulating the amount of phosphate in animal waste was established in 1996 in Korea and in European countries it is already mandatory to add phytase in the feeds of animals.

The addition of phytase in the feeds may greatly improve the productivity of livestock by enhancing the availability of some bioactive substances(phosphate, calcium and zinc etc.) which, otherwise chelate with phytictaine and lose their activity.

As the result, the use of feeds containing phytase in livestock can enhance the availibility of feeds and reduce the environmental contamination caused by phosphate.

For the aforementioned benefits, intensive studies about phytase including the effects of phytase on animals (L.G. Young et al., 1993, X.G. Lei et al., 1994, Z. Mroz et al., 1994) have been performed mainly in Europe (A.H.J. Ullah et al., 1994, K.C. Ehrich, 1994, C.S. Piddington, 1993). However, since phytase can cleave only a limited number of phosphates and it mostly produced by molds which have long growing period, it is uneconomical in terms of mass production. In addition, it is difficult to use the phytic acid as an additive for monogastric animals since it is unsuitable to their physiological mechanism.

SUMMARY OF THE INVENTION

Therefore, the inventor et al. have identified a novel phytase-producing microorganism among hundred kinds of molds, Actinomycetes, bacteria, etc., obtained from soils and barns throughout the country, in an effort to produce phytase having excellent enzymatic potency and shorten the production period compared with the conventional phytase. Because of the high enzymatic potency of this novel microorganism and its physiological

suitability to use for monogastric animals and short period of kproduction compared to those of conventional enzymes the inventor et al. have judged that this enzyme has novelty and they have completed this invention.

The object of this invention is to provide novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and phytase enzyme, which is suitable to use for monogastric animals with excellent properties and more shortened production period.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is electron microscope photograph (X 10,000) of *Bacillus sp.* DS11 (KCTC 0231BP) strain, a novel strain according to this invention,

Fig. 2 is a SDS-PAGE electrophoresis analysis of novel phytase produced by *Bacillus sp.* DS11 (KCTC 0231BP), A: precipitation of acetone, B: resource S, C: superose 12 HR 10/30

Fig. 3 is a graph measuring of novel phytase produced by *Bacillus sp.* DS11 (KCTC 0231BP), a novel strain, in accordance with EXPERIMENTAL EXAMPLE 2 of this invention.

DETAILED DESCRIPTION OF THIS INVENTION

This invention relates to a novel phytase produced from novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and particularly, to a novel strain *Bacillus sp.* DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.

This invention is described in detail as set forth hereunder:

This invention relates to novel strain *Bacillus sp.* DS11(KCTC 0231BP).

Further, this invention includes its novel enzyme phytase whose N-terminal amino acid sequence is expressed by the following [Sequence table 1] under the following conditions:

Optimal temperature: 65 ℃

Optimal pH: 7.0

Molecular weight: 43,000 dalton

Isoelectric point: 5.6

In addition, this invention includes a method to use said microorganism as feed additives.

This invention is described in more detail as set forth hereunder:

This invention relates to a novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and novel phytase produced from said strain. The procedure for isolating and identifying said novel microorganism is as follows:

[Isolation of novel microorganisms]

From several thousands of strains obtained from soils and in barns throughout the country, a strain was isolated which has excellent resolution in phytase screen plate containing 15g of D-glucose, 5 g of calcium phytate, 5g of NH_4NO_3, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4H_2O$ and agar 15 g at pH 7.0/ l .

[Identification of novel microorganism]

The morphological property of the strain isolated from the above procedure is as follows:

1) Morphological property

In the grams staining test of the strain cultivated in an optimal growth medium, the strain was proven to be gram-positive. Fig. 1, the picture of electron microscope shows that strain is a rod type of 0.8 ~ 1.8 ㎛ in cell size.

In the growth test of the thermally inactivated cells at 80 ℃, said strain has formed thermostable spores. Further, its catalase test in which it shows the positive response demonstrates that it coincides with the morphological property of *Bacillus sp.*

The physiological property of said strain is as follows:

2) Physiological property

The test results of physiological property of the strain are represented in the following table 1. As shown in the table 1, it is assumed that strain is a facultative microorganism which may be grow in both aerobic and anaerobic states. With the difference of grown strains found at 50 ℃ and pH 5.7 compared with *Bacillus pantothenticus,* the strain of this invention is a mutant derived from *Bacillus pantothenticus.*

Table 1.

Property		*Bacillus pantotheniticus*	Strain of this invention
Catalase		+	+
Anaerobic culture		+	+
V-p. test		-	+
Acid-forming	glucose	+	+
	L-arabinose	-	+
	D-xylose	-	-
	D-mannitol	-	+
Hydrolysis	Casein	d	+
	Gelatin	+	+
	Starch	+	+
Synthesis	Indole	-	-
	Dihydroacetone	-	-
Growth pH	6.8	+	+
	5.7	-	+
Concentration of growth salt (at NaCl)	2%	+	+
	5%	+	+
	7%	+	+
	10%	+	+
Growth temperature	5℃	-	-
	10℃	-	-
	30℃	+	+
	40℃	+	+
	50℃	-	+

Note) +: positive. -: negative. d: different from species.

The chemical property of the strain obtained as above is as follows:

3) The chemical property of strain

After harvesting the strain, the test results of their various properties (e.g., G + C content, fatty acid composition, Murein Type and main melaquinone) are represented in the following table 2.

As shown in the above table 2, it is noted that the strain of this invention is similar to *Bacillus pantotheniticus* in terms of G + C content, murein type and main melaquinone including fatty acid of strain but it seems to difficult to make a judgement that both strains are the same species. Thus, it seems that the strain of this invention is a mutant derived from *Bacillus pantothenticus*.

Table 2.

Property		*Bacillus pantotheniticus*	Strain of this invention
G + C content		36.9	45.6
Murein type		Mezo-DAP	Mezo-DAP
Main melaquinone		MK-7	MK-7
Fatty acid of strain (%)	14 : 0 ISO	4.71	1.96
	14 : 0	1.50	1.21
	15 : 0 ISO	19.34	18.72
	15 : 0 ANTEISO	37.95	38.51
	16 : 0 ISO	10.01	6.16
	16 : 0	9.77	9.77
	17 : 0 ISO	4.37	9.02
	17 : 0 ANTEISO	12.00	11.20
	18 : 0	x	2.03

By compiling above mentioned morphological, physiological and chemical properties, it is revealed that the strain of this invention belongs to *Bacillus sp.* from Bergys Manual of Systemic Bacteriology Vol. 2 (Williams & Wilkins Co., 1989). Therefore, the microorganism isolated was nominated as *Bacillus sp.* DS11 and deposited on February 1, 1996, to Korea Research Institute of Bioscience and Biotechnology Korean Collection for type cultures, Korean Patent Strain Deposit Associations located in #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea, with an accession number KCTC 0231BP.

This invention is described in more detail by the following examples, but the claims are not limited to these examples.

EXAMPLE 1: Production of novel phytase

To produce phytase, a medium containing 6% wheat bran, NH_2NO_3 0.04%, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% casein hydrolysate, 0.05% KH_2PO_4, 0.04% K_2HPO_4 and 0.2% $CaCl_2$, was adjusted to pH 6.5 and sterilized at 121 ℃ for 15 minutes.

Then, with the same medium composition, 1% seed culture cultivated in a flask at 37℃ for 12 hours was inoculated into the medium in order to produce the enzyme.

The potency of produced enzyme was measured as follows: By using a substrate, comprising 2 mM phytic acid sodium salts and 2mM $CaCl_2$ in 0.1 M Tris buffer solution (pH 7.0), the enzyme was reacted at 37℃ for 30 minutes to measure the amount of phosphoric acid generated. One unit of the enzymatic potency is equivalent to the enzymatic amount degrading 1 μ mol of inorganic phosphate per 1 minute. As a result of said measurement, the novel enzyme has the enzymatic potency of 0.3 unit per protein mg. The maximum amount of enzyme in fermentation yielding 0.6 unit/mg was obtained when 30 l of strains, a working volume, was charged to a 50 l

fermenter and cultivated at 37 ℃ for 48 hours under the following conditions: air influx - 0.8 vvm; stirring rate - 150 rpm. Compared with the enzymatic production amount of 0.3 unit/mg when phytase enzyme derived from *Aspergillus ficuum* is cultivated for 96 hours [Donna M. Gibson, 1987], it is well observed that the novel organism of this invention has a production amount twice higher.

EXPERIMENTAL EXAMPLE 1: Measurement of molecular weight for novel phytase

2 *l* of novel strain solution, cultivated according to the procedure as described in said Example, was centrifuged for 15 minutes. Then, the supernatant solution was saturated with 50% acetone to precipitate proteins and the crude enzyme solution passed through dialysis membrane was purified on a column containing phenyl sepharose CL-4B, Resource S and superose 12 HR 10/30 (all of which are manufactured by Pharmacia, Sweden) so as to isolate the phytase enzyme only. The analytical results by said column are represented in the attached drawing Fig. 2. Phytase enzyme was again on SDS-PAGE electrophoresis and the results show that the molecular weight of phytase produced by a novel strain is 43,000 dalton and isoelectric point is 5.6.

Further, the enzyme protein, so isolated, was used to determine the sequence of N-terminal amino acid using Protein/peptide Sequencer (Applied Biosystems, USA) and its results are represented in the following [Sequence table].

The sequencing No. 1 of [Sequence table] is the sequence of N-terminal amino acids of the phytase produced from a novel strain of this invention; the sequencing No. 2 is the sequence of the N-terminal amino acids of the phytase produced from E. coli [Arch. Biochem. Biophys. 303(1993)]; the sequencing No. 2 is the sequence of the N-terminal amino acids of the phytase produced from

Aspegillus ficuum [Prep. Biochem. 18(1988)].

From the results of [Sequence table], it is revealed that the phytase enzyme produced by *Bacillus sp.* DS11(KCTC 0231BP) of this invention is a novel enzyme.

EXPERIMENTAL EXAMPLE 2: Activity and stability of novel phytase to temperature and pH

The optimal temperature was proved to be 65℃ by using the enzyme isolated by the same method as described in the experimental example 2. To measure the thermal stability, the enzyme was left at each temperature for 10 minutes so as to assess the residual activity. As shown in the attached drawing Fig. 3 (3-1), the results show that when Ca^{2+} was not added, the activity began to decrease at 40 ℃ but in case of adding 5mM calcium ion, the activity was stable up to 70℃ and 50% of the activity at 75℃ was sustained.

From the aforementioned results, a novel phytase of this invention may be expected to a higher activity in the body of livestock. Therefore, it seems to be preferable that the feeds should be pelleted or extruded at more than 75℃ so as to use them as processing ones.

The results of phytase activity in the different conditions of pH are shown in the attached drawing Fig. 3 (3-2) and the optimal pH was 7.0. Further, to measure the stability on pH, the enzyme was left at various conditions of pH for 1 hour so as to assess the residual activity of phytase. Even under the acidic condition of below pH 4, the enzyme was proved to have higher enzymatic activity and from these results, it is judged that the novel phytase of this invention may be extremely stable under the acidic condition of stomach.

From the test results of the aforementioned temperatures and pH, it is noted that the novel phytase of this invention may be applicable as a feed additive of monogastric animals.

EXPERIMENTAL EXAMPLE 3: Influence of metal ion and inhibitor on enzyme activity

Influence of metal ion and inhibitor on enzyme activity is represented in the following table 3.

The results of table 3 show that the addition of 1mM EDTA inhibited the entire enzyme activity and when Cu^{2+}, Zn^{2+} and Mg^{2+} were added at the concentration of 5mM, about 50% of enzyme activity was reduced.

Table 3.

Additives	Concentration	
	1 mM	5 mM
Non-addition	100	100
$CuCl_2$	63	43
$ZnCl_2$	87	47
$MgCl_2$	95	49
$MnCl_2$	65	20
$LiCl_2$	95	100
$HgCl_2$	83	62
$CaCl_2$	99	116
$RbCl_2$	103	102
EDTA	7.5	7.6
PMSF	86	88

EXPERIMENTAL EXAMPLE 4: Influence of novel phytase added to feed for broiler on environmental contamination

To estimate the influence relation of availability and released amounts of phosphate when phytase is added, broilers were divided into three groups such as novel phytase group, soybean-extracted plant phytase group and mold

phytase group on the market(manufacturer: Sigma). Each of hatched-out 200 males of Avaachre broiler chicken was announced publicly for this experiment. The cultured medium containing phytase was ultrafiltrated and further concentrated at low temperature under vacuum and dried by lyophilizer. Each 500 unit of this lyophilized phytase was added to per kg of feed. The same amount of soybean phytase or mold phytase as above was also added to the feeds and the results were represented in the following table 4.

Table 4

Classification	Plant (soybean) phytase	Commercial mold phytase	Novel phytase
Phosphate contents in feeds (g/kg)	5.5	5.5	5.5
Intake of total feeds (g/kg)	2.7	2.7	2.7
Intake of phosphate (g/head)	15.1	15.2	15.1
Accumulation of phosphate (g/head)	7.1	7.8	8.8
Discharge of phosphate (g/head)	8.0	7.4	6.3
Absorption of phosphate (%)	47	51	58

The results of Table 4 revealed that when novel phytase was fed to monogastric animals, the phosphate contents released were lower than the soybean group or mold phytase group, since the phosphate availability in the former was higher than that of the latters; Namely, the activity of novel phytase was superior under acid-fast and acidic conditions and phytictaine within the grains was degraded in the intestine of animals by the novel phytase in more effective manner.

[Sequence table]

Sequencing No.: 1

Sequencing length: 15

Sequencing form: amino acid

Shape: straight chain

Sequencing type: protein

Sequence: Ser-Asp-Pro-Tyr-His-Phe-Thr-Val-Asn-Ala-Ala-Xaa-Glu-Thr-Glu

Sequencing No.: 2

Sequencing length: 11

Sequencing form: amino acid

Shape: straight chain

Sequencing type: protein

Sequence: Ser-Glu-Pro-Glu-Leu-Lys-Leu-Glu-Ala-Val-Val

Sequencing No.: 3

Sequencing length: 12

Sequencing form: amino acid

Shape: straight chain

Sequencing type: protein

Sequence: Pro-Ala-Ser-Arg-x-Gin-Ser-Ser-Cys-Asp-Thr-Val

WHAT IS CLAIMED IS :

1. Strain *Bacillus sp.* DS11(KCTC 0231BP).

2. The method to use strain *Bacillus sp.* DS11(KCTC 0231BP) as feed additives.

3. The phytase produced by *Bacillus sp.* in claim 1 has the following characterization:

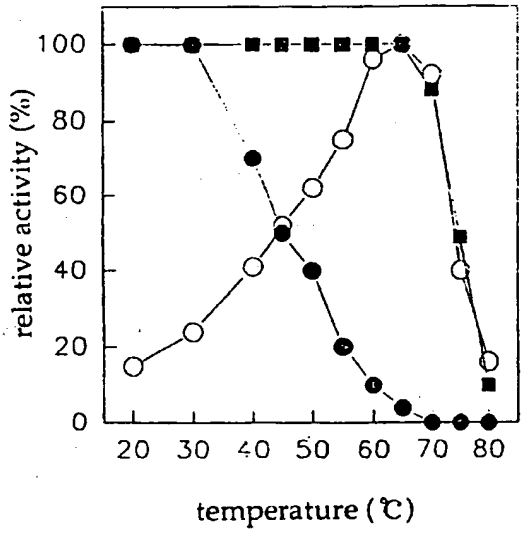
Optimal temperature	65 ℃
Optimal pH	7.0
Molecular weight	43,000 dalton
Isoelectric point	5.6
Sequence of N-terminal amino acid	No. 1 based on [sequencing table]

4. The method to use a phytase produced by strain *Bacillus sp.* DS11(KCTC 0231BP) as feed additives according to claim 3.

FIG. 1

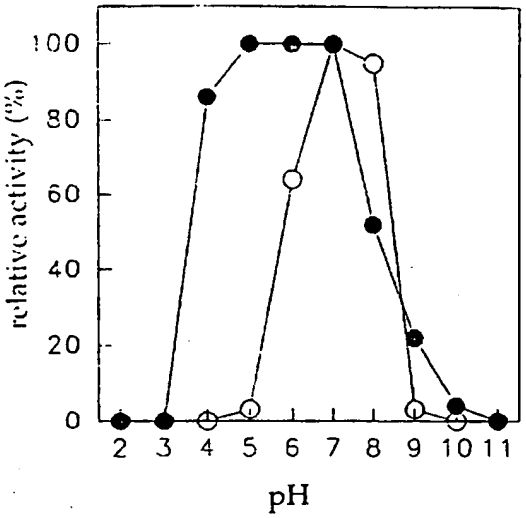
FIG. 2

FIG. 3-1



-○- ; optimal temperature
-●- ; stability in the non-existance of Ca^{2+}
-■- ; stability in the existance of 5mM Ca^{2+}

FIG.3-2



-○- : optimal pH
-●- ; stability

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00040

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 1/20, 9/16; A 23 K 1/165 // (C 12 N 1/20; C 12 R 1:07)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 1/20, 9/16; A 23 K 1/165

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, Vol.18, No.257 (C-1200), 1994, Kokai-No. 6-38745 (ZENKOKU NOGYO KYODO KUMIAI RENGOKAI).	1-3
A	WO 94/19 471 A1 (THE FINNISH NATIONAL PUBLIC HEALTH INSTITUTE) 01 September 1994 (01.09.94), claims 1-15.	1-3

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Date of the actual completion of the international search	Date of mailing of the international search report
16 May 1997 (16.05.97)	02 June 1997 (02.06.97)

Name and mailing address of the ISA/AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna	Wolf
Facsimile No. 1/53424/535	Telephone No. 1/53424/133

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 97/00040

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9419471	01-09-94	AU A1 61426/94	14-09-94
		CA AA 2156425	01-09-94
		CN A 1120853	17-04-96
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